AMENDMENTS TO THE SPECIFICATION:

Please amend the specification as follows:

Please delete the paragraph spanning line 15 of page 15 through line 14 of page 16 and insert the following therefor:

In the present invention we have established for the first time the presence of lymphatic vessels, by using molecular *in situ* hybridization of tadpoles at various embryonic stages with molecular probes, known to mark lymphatic vessels in the mouse. Therefore, the *Xenopus* orthologue sequences of murine Prox-1 (termed from hereon as "x-Prox-1") and VEGF receptor-3 (termed "x-VEGFR-3") were cloned. Hybridization was performed using: (i) a 950 nucleotide (nct) fragment of the x-Prox-1 cDNA (nucleotide 190 till 1139 relative to the ATG start codon), obtained by RT-PCR amplification of tadpole RNA using the following primers (downstream primer: 5'GGG GAA AAG TCA AAT GTT CTC CG 3' (SEQ ID NO: 1); upstream primer: 5' AGT TGG CGA ATG GGC TTA GC 3' (SEQ ID NO: 2), derived from NCBI# AB008773 gene sequence); (ii) a 1552 bps fragment of the x-VEGFR-3 cDNA, obtained by RT-PCR amplification of tadpole RNA using the following primers (downstream primer: 5' GTT TTC ATC TGA AAA GAA TGA TGC ATG GAT G 3' (SEQ ID NO: 3) ; upstream primer: 5' ACT TGG AAA AGT CCT GGG TCG TGG AGC 3' (SEQ ID NO: 4), derived from the genbank EST # BM261245). Whole mount *in situ* hybridization with digoxigenin-labeled antisense RNA probes was carried out according to previously reported protocols [55,56]. Briefly, antisense RNA probes were synthesized using digoxigenin labeling mix (Boehringer Mannheim). Unincorporated nucleotides were removed by gel filtration

through G-50 Sephadex columns (Mini Quick Spin Columns, Boehringer Mannheim). Embryos from different developmental stages were collected and fixed for 2 h in MEMFA fix and stored in 100% ethanol -20 C until further use. Following rehydration, the embryos were treated with proteinase K (10 μg/ml) to increase sensitivity and triethanolamine/acetic anhydride to inhibit endogenous phosphatases. Hybridizations with digoxigenin- labeled antisense RNA probes (0.5μg/ml) were carried out at 60°C overnight. Sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) were used to detect the digoxigenin-labeled probes. Color development was performed using BM purple (Boehringer Mannheim). Embryos were bleached to diminish pigmentation and cleared in 100% ethanol before photographic documentation. After staining, tadpoles were cross-sectioned and counterstained. In addition, we also used an anti-murine Prox-1 antibody (which crossreacts with x-Prox-1) to immunolabel lymphatic vessels on cross-sections. This analysis revealed the presence of Prox-1 positive vascular structures both rostrally and caudally in the stage 35-36 tadpole. In addition, the anlage of a lymphatic heart could be clearly visualized in the jugular region, dorsally of the promesonephric tubules. As in the mouse, expression of Prox-1 was found to be first confined to the external region of the cardinal vein, indicating lymphatic commitment of venous endothelial cells. At later stages (e.g. stage 40), the ventral and dorsal caudal lymphatic vessels in the tail of the tadpole also stained positive, when *in situ* hybridized using the x-Prox-1 or x-VEGFR-3 probe. The ventral caudal lymphatic vessel lied ventrally of the posterior cardinal vein – their close proximity further suggesting that the lymphatic vessel derived from the vein.

Please delete the paragraphs spanning line 19, of page 16 through line 25 of page 18 and insert the following therefor:

The first method involves the overexpression of x-VEGF-C, a well-known key molecule regulating lymphangiogenesis in other animals. Overexpression of VEGF-C is known to stimulate lymphangiogenesis in health and disease. We employed tadpole RNA to amplify by RT-PCR (using the downstream primer 5' GAG GCA CCA CCG GAT TTG ACA CCA 3' (SEQ ID NO: 5) and upstream primer 5' GTG GTA GTG TTG CTG GCA GGG AAC GTC 3' (SEQ ID NO: 6)) the open reading frame, encoding the mature form of x-VEGF-C (sequence information public available as EST NCBI# CA973641). This sequence, preceded by an ATG initiation codon, a Kozak and signal peptide sequence was cloned into the $pCS2^+$ expression vector, containing the ubiquitously and constitutively active CMV promoter. *Xenopus* eggs were obtained and fertilized by standard methods [57], de-jellied with 2% cysteine (pH 8.0), rinsed, and placed in 1 xMMR + 6% Ficoll for injections. In vitro transcribed x-VEGF-C RNA (1.0 ng in 5 nl volume) was then injected into the ventral marginal blastomeres of either 8-cell stage or 16-cell stage *Xenopus* embryos to target the expression of VEGF-C to the putative jugular region of developing *Xenopus* embryos. Embryos were staged according to the table of *Xenopus laevis* development [58]. Labeling of the lymphatic endothelial cells by *in situ* Prox-1 hybridization of transgenic tadpoles at stage 33-34 revealed that the lymphatic sac in the jugular region was dilated and hyperplastic, with more numerous Prox-1 positive lymphatic endothelial cells than in embryos after control DNA injection.

In a second example 25 ng of Prox-1 antisense morpholino oligonucleotides (Prox-1 MOs) was microinjected in a 1-cell stage *Xenopus*, in order to investigate the influence on the development of the lymphatic system. Prox1 is known to be expressed exclusively in lymphatic but not in blood vascular endothelial cells. *Xenopus laevis* embryos were obtained by artificial fertilization using standard techniques and sexually mature adult frogs purchased from Nasco Biology (Fort Atkinson, WI). The Prox-1 MOs, 5'-CAGGCATCACTGGACTGTTATTGTG-3' <u>(SEQ ID NO: 9)</u>, were purchased from Gene Tools (Corvallis, OR) and designed around the ATG codon. As ATG-specific morpholinos lower translation – but not transcription – of the target gene, we used whole embryo mount *in situ* hybridisation for Prox1 to visualize the defects in lymphatic vascular development. The 5' nucleotide sequence of Prox-1 was obtained using 5' RACE using primers based on published *Xenopus laevis* cDNA (GenBank # AB008773). To visualise Prox-1 expression pattern, embryos were collected at different developmental stages (according to Nieuwkoop and Faber), fixed and submitted to whole-mount *in situ* hybridisation according Harland (Harland, 1991), with the exception that BM purple was used as a substrate. The Prox-1 digoxigenin-labeled riboprobe used was constructed from a 950 bp cDNA fragment (GenBank # AB008773), which was cloned into pGEMTeasy and linearised with AatII to get SP6 antisense probe. After in situ hybridisation the embryos where bleached, cleared and stored in glycerol until analysed. Analysis was performed using a Zeiss Axioplan 2 imaging microscope equipped with a Axiocam HrC camera and the KS300 morphometry software. The total ISH-region was divided into 3 different regions where the first region represents

the ventral side the tail, a second region where the dorsal migration of prox-1 positive cells takes place and finally a third region which represents the dorsal side. For all regions, length of positive staining was measured, followed by segmentation of the ISH-area by hue, lightness and saturation for defining the true stained area. A value of migration is given by the cell that travelled furthest across the tail. In non-morpholino treated *Xenopus* we observed that the normal lymphatic system starts developing at the jugular region of the tadpole around developmental stage 29/30. Apart from expression in non-lymphatic tissues such as the pronephros and the eye, the Prox-1 expression at this stage seems to be restricted to the jugular region. At the following stage (31-32), the staining in the jugular region is expanded to what will become the future lymphsacs. The region of the future anterior lymph hearts as well as the ventral caudal region show intense Prox-1 positive signal too. By stage 33/34 the prox positive cells in the caudal region start the formation of the ventral caudal lymphatic vessel. Prox-1 positive lymphatic cells are migrating from the ventral to the dorsal region and this event is culminating at stage 35/36 where the highest amount of migrating cells can be accounted for. The formation of what eventually will become the dorsal lymphatic vessel is also initiated at this stage. By stage 37-38 a majority of the prox positive cells have reached the dorsal side where they are contributing to the formation of dorsal caudal lymphatic vessels. In Prox-1 morpholino treated *Xenopus* (designated as Prox-1 morphants or Prox-1 knockdowns) although we observed that Prox-1 staining of the anterior region is clearly reduced we have in particular focused on the analysis of the tail region of the tadpoles. In the tail of the Prox-1 morphants dorsal migration of Pro-1

positive cells is clearly impaired. This is most apparent for stage 35/36 where migration is significantly reduced with about 55%. Though to a lesser degree, the reduction is also significantly reduced for stage 37/38. The total Prox-1 positive area is also significantly reduced in about 60% of the Prox-1 morphants at stage 33/34, 50% for stage 35/36 and 35% for stage 37/38 as compared to control tadpoles. This is in agreement with the phenotype Prox-1 null mice where the development of the lymphatic vasculature has been found to be affected due to an arrest of the budding and migration of the prox positive structures (Wigle JT and Oliver G (1999) *Cell*, 98(6):769-78. Knockdown of Prox1 also impaired the formation of the lymph hearts and the rostral lymphatic structures. Interestingly, manifest edema in the head and anterior trunk developed in most Prox1 knockdown embryos beyond stage 45, when the lymphatic circulation is functional in control tadpoles. Upon macroscopic inspection or microscopic analysis, we could not detect any signs of extravasation of red blood cells, further indicating that the swelling represents lymphedema, resulting from insufficient lymph drainage. The lymphatic defects were not attributable to defects in cardiogenesis (looping, septation and beating of the heart, and blood circulation were all normal in Prox1 morfants, neither were they secondary to abnormal angiogenesis. Indeed, when scoring angiogenesis by counting the number of Msr-positive intersomitic vessels (ISVs) in the anterior trunk and measuring the Msr-positive area in the posterior trunk, development of blood vessels was not affected in Prox1 morphants. Msr is the mesenchyme-associated serpentine receptor, which is a homologue of the G protein-coupled receptor

angiontensin-receptor related protein, and is specifically expressed by blood vascular endothelial cells.